

Date March 26, 1997

**Box Patent Application**  
**Assistant Commissioner for Patents**  
**Washington DC 20231**

Sir:

Transmitted herewith for filing is the patent application of  
Inventor: Sietse MOSSELMAN and Rein DIJKEMA



- [X] Specification and claims. (60 pages)
- [X] Five (5) sheets of drawing(s).
- [X] Preliminary Amendment
- [X] Information Disclosure Statement with PTO 1449
- [X] A filing fee calculated as shown below:

FOR:	NO. FILED	NO. EXTRA	RATE	FEE
BASIC FEE				\$ 770.00
TOTAL CLAIMS	20 - 18 =	0	X \$ 22	\$ .00
INDEP CLAIMS	3 - 1 =	0	X \$ 80	\$ .00
[ ] MULTIPLE DEPENDENT CLAIMS PRESENTED			+ \$260	\$ .00
			TOTAL	\$ 770.00

- [X] Please charge my Deposit Account No. 02-2334 in the amount of \$770.00 to cover the filing fee and [ ] assignment recordation. A duplicate of this sheet is attached.
- [X] The Commissioner is hereby authorized to charge payment for the following fees associated with this communication or credit any overpayment to Deposit Account No. 02-2334. A duplicate copy of this sheet is attached.
  - [X] Any additional filing fees required under 37 CFR 1.16.
- [X] The Commissioner is hereby authorized to charge payment for any additional fees associated with this communication or credit any overpayment to Deposit Account No. 02-2334. A duplicate copy of this sheet is attached.
  - [X] Any patent application processing fees under 37 CFR 1.17.
  - [X] Any filing fees under 37 CFR 1.16 for presentation of extra claims.

Respectfully submitted,  
AKZO NOBEL N.V.

*Mary E. Gormley*

By: Mary E. Gormley  
Registration No. 34,409

1300 Piccard Drive #206  
Rockville, Maryland 20850-4373  
Tel: (301) 948-7400  
MEG/sp  
Enclosures  
37moslmm.apl

Express Mail No. EM193123489 US



In re the application of:

Serial Number: To be assigned      Group Art Unit: To be assigned

Filed: Concurrently herewith      Examiner: To be assigned

For: NOVEL ESTROGEN RECEPTOR

Corresponding to: European patent application Nos. 96200820.7,  
filed March 26, 1996 and 96203384.3, filed  
November 22, 1996

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents  
Washington, D.C. 20231

March 26, 1997

Sir:

Prior to the calculation of the fee in the above-identified application, please make the following amendments:

IN THE CLAIMS:

1. (amended) An isolated [Isolated] DNA encoding a protein having an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said protein exhibits at least 80% homology with the amino acid sequence shown in SEQ ID NO:3, and the amino acid sequence of said ligand-binding domain of said protein exhibits at least 70% homology with the amino acid sequence shown in SEQ ID NO:4.

2. (amended) The isolated [Isolated] DNA according to [claims] claim 1, [characterized in that] wherein the amino acid sequence of said DNA-binding domain of said protein exhibits at least 90%[, preferably 95%, more preferably 98%, most preferably 100%] homology with the amino acid sequence shown in SEQ ID NO:3.

Express Mail No.  
EM193123489US

[illegible]

3. (amended) The isolated [Isolated] DNA according to claim 1, [claims 1 or 2, characterized in that] wherein the amino acid sequence of said ligand-binding domain of said protein exhibits at least 75%[, preferably 80%, more preferably 90%, most preferably 100%] homology with the amino acid sequence shown in SEQ ID NO:4.

4. (amended) The isolated [Isolated] DNA according to claim 1 [claims 1 to 3], wherein said DNA [encoding] encodes a protein comprising [the] an amino acid sequence selected from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.

5. (amended) The isolated [Isolated] DNA according to claim 1 [claims 1 to 4, characterized in that] said DNA comprises [the] a nucleic acid sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:20 or SEQ ID NO:24.

6. (amended) A recombinant expression vector comprising the DNA according to claim 1 [any of the claims 1 to 5].

7. (amended) A cell transfected with DNA according to claim 1 [claims 1 to 5 or an expression vector according to claim 6].

8. (amended) [A] The cell according to claim 7, which is a stable transfected cell line [which] that expresses [the] a steroid receptor protein according to any of the claims 9 to 11.

9. (amended) A protein [Protein] encoded by DNA according to claim 1 [claims 1 to 5 or an expression vector according to claim 6].

10. (amended) The protein [Protein] according to claim 9, said protein comprising [the] an amino acid sequence selected

from the group consisting of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.

11. (amended) A chimeric [Chimeric] protein having an N-terminal domain, a DNA-binding domain, and a ligand-binding domain, [characterized in that] wherein at least one of said domains of said chimeric protein originates from a protein according to claim 9 [claims 9 or 10], and at least one of the other domains of said chimeric protein originates from another receptor protein from the nuclear receptor superfamily, provided that the DNA-binding domain and the ligand-binding domain of said chimeric protein [originates] originate from different proteins.

12. (amended) A DNA that encodes [encoding] a protein according to claim 11.

Please cancel claim 13 without prejudice or disclaimer of the subject matter thereof.

14. (amended) A method for identifying functional ligands for the protein according to claim 9 [claims 9 to 11], said method comprising the steps of

- a) introducing into a suitable host cell 1) DNA according to claim 1 [claims 1 to 5 or 12], and 2) a suitable reporter gene functionally linked to an operative hormone response element (HRE), said HRE being able to be activated by the DNA-binding domain of the protein encoded by said DNA;
- b) bringing the host cell from step a) into contact with potential ligands which will possibly bind to the ligand-binding domain of the protein encoded by said DNA from step a);
- c) monitoring the expression of the protein encoded by said reporter gene of step a).

Please add the following new claims 15-18:

-- 15. The isolated DNA according to claim 2, wherein the amino acid sequence of the DNA-binding domain is shown in SEQ ID NO: 3. --

-- 16. The isolated DNA according to claim 3, wherein the amino acid sequence of said ligand-binding domain is shown in SEQ ID NO: 4. --

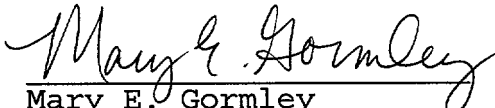
-- 17. A cell transfected with the expression vector according to claim 6. --

-- 18. A protein encoded by the expression vector of claim 6.  
--

REMARKS

It is believed that claims 1-18 recite a patentable improvement in the art. Favorable action is solicited. In the event any fees are required with this paper, please charge our Deposit Account No. 02-2334.

Respectfully submitted,

  
Mary E. Gormley  
Attorney for Applicants  
Registration No. 34,409

AKZO NOBEL N.V.  
1300 Piccard Drive, Suite 206  
Rockville, Maryland 20850-4373  
Tel: (301) 948-7400  
Fax: (301) 948-9751

MEG:ms

37moslmm.pre

Express Mail No.  
EM193123489US



5/

A

## Novel estrogen receptor

This invention relates to the field of receptors belonging to the superfamily of nuclear hormone receptors, in particular to steroid receptors. The invention relates to DNA encoding a novel steroid receptor, the preparation of said receptor, the receptor protein, and the uses thereof.

Steroid hormone receptors belong to a superfamily of nuclear hormone receptors involved in ligand-dependent transcriptional control of gene expression. In addition, this superfamily consists of receptors for non-steroid hormones such as vitamine D, thyroid hormones and retinoids (Giguère et al, Nature 330, 624-629, 1987; Evans, R.M., Science 240, 889-895, 1988). Moreover, a range of nuclear receptor-like sequences have been identified which encode socalled 'orphan' receptors: these receptors are structurally related to and therefore classified as nuclear receptors, although no putative ligands have been identified yet (B.W. O'Malley, Endocrinology 125, 1119-1170, 1989; D.J. Mangelsdorf and R.M. Evans, Cell, 83, 841-850, 1995).

The superfamily of nuclear hormone receptors share a modular structure in which six distinct structural and functional domains, A to F, are displayed (Evans, Science 240, 889-895, 1988). A nuclear hormone receptor is characterized by a variabel N-terminal region (domain A/B), followed by a centrally located, highly conserved DNA-binding domain (hereinafter referred to as DBD; domain C), a variable hinge region (domain D), a conserved ligand-binding domain (herein after referred to as LBD; domain E) and a variable C-terminal region (domain F).

The N-terminal region, which is highly variable in size and sequence, is poorly conserved among the different members of

the superfamily. This part of the receptor is involved in the modulation of transcription activation (Bocquel et al, Nucl. Acid Res., 17, 2581-2595, 1989; Tora et al, Cell 59, 477-487, 1989).

5       The DBD consists of approximately 66 to 70 amino acids and is responsible for DNA-binding activity: it targets the receptor to specific DNA sequences called hormone responsive elements (hereinafter referred to as HRE) within the transcription control unit of specific target genes on the  
10       chromatin (Martinez and Wahli, In 'Nuclear Hormone Receptors', Acad. Press, 125-153, 1991).

20       The LBD is located in the C-terminal part of the receptor and is primarily responsible for ligand binding activity. In this way, the LBD is essential for recognition and binding of the hormone ligand and, in addition possesses a transcription activation function, thereby determining the specificity and selectivity of the hormone response of the receptor. Although moderately conserved in structure, the LBD's are known to vary considerably in homology between the individual members of the  
25       nuclear hormone receptor superfamily (Evans, Science 240, 889-895, 1988; P.J. Fuller, FASEB J., 5, 3092-3099, 1991; Mangelsdorf et al, Cell, Vol. 83, 835-839, 1995).

30       Functions present in the N-terminal region, LBD and DBD operate independently from each other and it has been shown that these domains can be exchanged between nuclear receptors (Green et al, Nature, Vol. 325, 75-78, 1987). This results in chimeric nuclear receptors, such as described for instance in WO-A-8905355.

35       When a hormone ligand for a nuclear receptor enters the cell by diffusion and is recognized by the LBD, it will bind to the specific receptor protein, thereby initiating an allosteric alteration of the receptor protein. As a result of

this alteration the ligand/receptor complex switches to a transcriptionally active state and as such is able to bind through the presence of the DBD with high affinity to the corresponding HRE on the chromatin DNA (Martinez and Wahli, 'Nuclear Hormone Receptors', 125-153, Acad. Press, 1991). In this way the ligand/receptor complex modulates expression of the specific target genes. The diversity achieved by this family of receptors results from their ability to respond to different ligands.

The steroid hormone receptors are a distinct class of the nuclear receptor superfamily, characterized in that the ligands are steroid hormones. The receptors for glucocorticoids (GR), mineralcorticoids (MR), progestins (PR), androgens (AR) and estrogens (ER) are classical steroid receptors. Furthermore, the steroid receptors have the unique ability upon activation to bind to palindromic DNA sequences, the so-called HRE's, as homodimers. The GR, MR, PR and AR recognize the same DNA sequence, while the ER recognizes a different DNA sequence. (Beato et al, Cell, Vol. 83, 851-857, 1995). After binding to DNA, the steroid receptor is thought to interact with components of the basal transcriptional machinery and with sequence-specific transcription factors, thus modulating the expression of specific target genes.

Several HRE's have been identified, which are responsive to the hormone/receptor complex. These HRE's are situated in the transcriptional control units of the various target genes such as mammalian growth hormone genes (responsive to glucocorticoid, estrogen, testosterone), mammalian prolactin genes and progesterone receptor genes (responsive to Estrogen), avian ovalbumin genes (responsive to progesterone), mammalian metallothionein gene (responsive to glucocorticoid)



and mammalian hepatic  $\alpha_2\mu$ -globulin gene (responsive to estrogen, testosterone, glucocorticoid).

5       The steroid hormone receptors have been known to be involved in embryonic development, adult homeostasis as well as organ physiology. Various diseases and abnormalities have been ascribed to a disturbance in the steroid hormone pathway. Since the steroid receptors exercise their influence as  
10 hormone-activated transcriptional modulators, it can be anticipated that mutations and defects in these receptors, as well as overstimulation or blocking of these receptors might be the underlying reason for the altered pattern. A better knowledge of these receptors, their mechanism of action and of  
15 the ligands which bind to said receptor might help to create a better insight in the underlying mechanism of the hormone signal transduction pathway, which eventually will lead to better treatment of the diseases and abnormalities linked to altered hormone/receptor functioning.

20       For this reason cDNA's of the steroid and several other nuclear receptors of several mammals, including humans, have been isolated and the corresponding amino acid sequences have been deduced, such as for example the human steroid receptors PR, ER, GR, MR, and AR, the human non-steroid  
25 receptors for vitamin D, thyroid hormones, and retinoids such as retinol A and retinoic acid. In addition, cDNA's encoding well over 100 mammalian orphan receptors have been isolated, for which no putative ligands are known yet (Mangelsdorf et al, Cell, Vol.83, 835-839, 1995). However, there is still a  
30 great need for the elucidation of other nuclear receptors, in order to unravel the various roles these receptors play in normal physiology and pathology.

The present invention provides for such a novel nuclear receptor. More specific, the present invention provides for novel steroid receptors, having estrogen mediated activity. Said novel steroid receptors are novel estrogen receptors, which are able to bind and be activated by, for example, estradiol, estrone and estriol.

According to the present invention it has been found that a novel estrogen receptor is expressed as an 8 kb transcript in human thymus, spleen, peripheral blood lymphocytes (PBLs), ovary and testis. Furthermore, additional transcripts have been identified. Another transcript of approximately 10 kb was identified in ovary, thymus and spleen. In testis, an additional transcript of 1.3 kb was detected. These transcripts are probably generated by alternative splicing of the gene encoding the novel estrogen receptor according to the invention.

Cloning of the cDNA's encoding the novel estrogen receptors according to the invention revealed that several splicing variants of said receptor can be distinguished. At the protein level, these variants differ only at the C-terminal part.

cDNA encoding an ER has been isolated (Green, et al, Nature 320, 134-139, 1986; Greene et al, Science 231, 1150-1154, 1986), and the corresponding amino acid sequence has been deduced. This receptor and the receptor according to the present invention, however, are distinct, and encoded for by different genes with different nucleic acid sequences. Not only do the ER of the prior art (hereinafter referred to as classical ER) and the ER according to the present invention differ in amino acid sequence, they also are located on different chromosomes. The gene encoding the classical ER is located on chromosome 6, whereas the gene encoding the ER according to the invention was found to be located on chromosome 14. The ER according to the invention furthermore

distinguishes itself from the classical receptor in differences in tissue distribution, indicating that there may be important differences between these receptors at the level of estrogenic signalling.

5        In addition, two orphan receptors,  $ERR\alpha$  and  $ERR\beta$ , having an estrogen receptor related structure have been described (Giguère et al, Nature 331, 91-94, 1988). These orphan  
10        receptors, however, have not been reported to be able to bind estrodial or any other hormone that binds to the classical ER, and other ligands which bind to these receptors have not been  
15        found yet. The novel estrogen receptor according to the invention distinguishes itself clearly from these receptors since it was found to bind estrogens.

20        The fact that a novel ER according to the invention has been found is all the more surprising, since any suggestion  
25        towards the existence of additional estrogen receptors was absent in the scientific literature: neither the isolation of the classical ER nor the orphan receptors  $ERR\alpha$  and  $ERR\beta$   
30        suggested or hinted towards the presence of additional estrogen receptors such as the receptors according to the invention. The identification of additional ER's could be a  
35        major step forward for the existing clinical therapies, which are based on the existence of one ER and as such ascribe all estrogen mediated abnormalities and/or diseases to this one  
40        receptor. The receptors according to the invention will be useful in the development of hormone analogs that selectively activate either the classical ER or the novel estrogen  
45        receptor according to the invention. This should be considered as one of the major advantages of the present invention.

50        Thus, in one aspect, the present invention provides for isolated cDNA encoding a novel steroid receptor. In

particular, the present invention provides for isolated cDNA encoding a novel estrogen receptor.

According to this aspect of the present invention, there is provided an isolated DNA encoding a steroid receptor protein having an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said receptor protein exhibits at least 80% homology with the amino acid sequence shown in SEQ ID NO:3, and the amino acid sequence of said ligand-binding domain of said receptor protein exhibits at least 70% homology with the amino acid sequence shown in SEQ ID NO:4.

In particular, the isolated DNA encodes a steroid receptor protein having an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said receptor protein exhibits at least 90%, preferably 95%, more preferably 98%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:3.

More particularly, the isolated DNA encodes a steroid receptor protein having an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said ligand-binding domain of said receptor protein exhibits at least 75%, preferably 80%, more preferably 90%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:4.

A preferred isolated DNA according to the invention encodes a steroid receptor protein having the amino acid sequence shown in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.

A more preferred isolated DNA according to the invention is an isolated DNA comprising a nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:20 or SEQ ID NO:24.

The DNA according to the invention may be obtained from cDNA. Alternatively, the coding sequence might be genomic DNA, or prepared using DNA synthesis techniques.

5 The DNA according to the invention will be very useful for in vivo expression of the novel receptor proteins according to the invention in sufficient quantities and in substantially pure form.

10 In another aspect of the invention, there is provided for a steroid receptor comprising the amino acid sequence encoded by the above described DNA molecules.

15 The steroid receptor according to the invention has an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said receptor exhibits at least 80% homology with the amino acid sequence shown in SEQ ID NO:3, and the amino acid sequence of said ligand-binding domain of said receptor exhibits at least 70% homology with the amino acid sequence  
20 shown in SEQ ID NO:4.

In particular, the steroid receptor according to the invention has an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said receptor exhibits at least 90%,  
25 preferably 95%, more preferably 98%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:3.

More particular, the steroid receptor according to the invention has an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said  
30 ligand-binding domain of said receptor exhibits at least 75%, preferably 80%, more preferably 90%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:4.

It will be clear for those skilled in the art that also steroid receptor proteins comprising combined DBD and LBD

preferences and DNA encoding such receptors are subject of the invention.

Preferably, the steroid receptor according to the invention comprises an amino acid sequence shown in SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.

Also within the scope of the present invention are steroid receptor proteins which comprise variations in the amino acid sequence of the DBD and LBD without losing their respective DNA-binding or ligand-binding activities. The variations that can occur in those amino acid sequences comprise deletions, substitutions, insertions, inversions or additions of (an) amino acid(s) in said sequence, said variations resulting in amino acid difference(s) in the overall sequence. It is well known in the art of proteins and peptides that these amino acid differences lead to amino acid sequences that are different from, but still homologous with the native amino acid sequence they have been derived from.

Amino acid substitutions that are expected not to essentially alter biological and immunological activities, have been described in for example Dayhof, M.D., Atlas of protein sequence and structure, Nat. Biomed. Res. Found., Washington D.C., 1978, vol. 5, suppl. 3. Amino acid replacements between related amino acids or replacements which have occurred frequently in evolution are, inter alia Ser/Ala, Ser/Gly, Asp/Gly, Arg/Lys, Asp/Asn, Ile/Val. Based on this information Lipman and Pearson developed a method for rapid and sensitive protein comparison (Science 227, 1435-1441, 1985) and determining the functional similarity between homologous polypeptides.

Variations in amino acid sequence of the DBD according to the invention resulting in an amino acid sequence that has at least 80% homology with the sequence of SEQ ID NO:3 will lead to receptors still having sufficient DNA binding activity. Variations in amino acid sequence of the LBD according to the

invention resulting in an amino acid sequence that has at least 70% homology with the sequence of SEQ ID NO:4 will lead to receptors still having sufficient ligand binding activity.

Homology as defined herein is expressed in percentages, determined via PCGENE. Homology is calculated as the percentage of identical residues in an alignment with the sequence according to the invention. Gaps are allowed to obtain maximum alignment.

Comparing the amino acid sequences of the classical ER and the ER's according to the invention revealed a high degree of similarity within their respective DBD's. The conservation of the P-box (amino acids E-G-X-X-A) which is responsible for the actual interactions of the classical ER with the target DNA element (Zilliacus et al., Mol.Endo. 9, 389, 1995; Glass, End.Rev. 15, 391, 1994), is indicative for a recognition of estrogen responsive elements (ERE's) by the ER's according to the invention. The receptors according to the invention indeed showed ligand-dependent transactivation on ERE-containing reporter constructs. Therefore, the classical ER and the novel ER's according to the invention may have overlapping target gene specificities. This could indicate that in tissues which co-express both respective ER's, these receptors compete for ERE's. The ER's according to the invention may regulate transcription of target genes differently from classical ER regulation or could simply block classical ER functioning by occupying estrogen responsive elements. Alternatively, transcription might be influenced by heterodimerization of the different receptors.

Thus, a preferred steroid receptor according to the invention comprises the amino acid sequence E-G-X-X-A within the P box of the DNA binding domain, wherein X stands for any

amino acid. Also within the scope of the invention is isolated DNA encoding such a receptor.

5       Methods to prepare the receptors according to the invention are well known in the art (Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989). The most practical approach is to produce these receptors by expression of the DNA encoding the  
10       desired protein.

15       A wide variety of host cell and cloning vehicle combinations may be usefully employed in cloning the nucleic acid sequence coding for the receptor of the invention. For example, useful cloning vehicles may include chromosomal, non-chromosomal and synthetic DNA sequences such as various known bacterial plasmids and wider host range plasmids and vectors derived from combinations of plasmids and phage or virus DNA. Useful hosts may include bacterial hosts, yeasts and other fungi, plant or animal hosts, such as Chinese Hamster Ovary (CHO) cells or monkey cells and other hosts.  
20

25       Vehicles for use in expression of the ligand-binding domain of the present invention will further comprise control sequences operably linked to the nucleic acid sequence coding for the ligand-binding domain. Such control sequences generally comprise a promoter sequence and sequences which regulate and/or enhance expression levels. Furthermore an origin of replication and/or a dominant selection marker are often present in such vehicles. Of course control and other sequences can vary depending on the host cell selected.

30       Techniques for transforming or transfecting host cells are quite known in the art (see, for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1989).



Recombinant expression vectors comprising the DNA of the invention as well as cells transformed with said DNA or said expression vector also form part of the present invention.

5

In a further aspect of the invention, there is provided for a chimeric receptor protein having an N-terminal domain, a DNA-binding domain, and a ligand-binding domain, characterized in that at least one of the domains originates from a receptor protein according to the invention, and at least one of the other domains of said chimeric protein originates from another receptor protein from the nuclear receptor superfamily, provided that the DNA-binding domain and the ligand-binding domain of said chimeric receptor protein originate from different proteins.

10

15

In particular, the chimeric receptor according to the invention comprises the LBD according to the invention, said LBD having an amino acid sequence which exhibits at least 70% homology with the amino acid sequence shown in SEQ ID NO:4. In that case the N-terminal domain and DBD should be derived from another nuclear receptor, such as for example PR. In this way a chimeric receptor is constructed which is activated by a ligand of the ER according to the invention and which targets a gene under control of a progesterone responsive element. The chimeric receptors having a LBD according to the invention are useful for the screening of compounds to identify novel ligands or hormone analogs which are able to activate an ER according to the invention.

20

25

In addition, chimeric receptors comprising a DBD according to the invention, said DBD having an amino acid sequence exhibiting at least 80% homology with the amino acid sequence shown in SEQ ID NO:3, and a LBD and, optionally, an N-terminal domain derived from another nuclear receptor, can be successfully used to identify novel ligands or hormone analogs

30

for said nuclear receptors. Such chimeric receptors are especially useful for the identification of the respective ligands of orphan receptors.

Since steroid receptors have three domains with different functions, which are more or less independent, it is possible that all three functional domains have been derived from different members of the steroid receptor superfamily.

Molecules which contain parts having a different origin are called chimeric. Such a chimeric receptor comprising the ligand-binding domain and/or the DNA-binding domain of the invention may be produced by chemical linkage, but most preferably the coupling is accomplished at the DNA level with standard molecular biological methods by fusing the nucleic acid sequences encoding the necessary steroid receptor domains. Hence, DNA encoding the chimeric receptor proteins according to the invention are also subject of the present invention.

Such chimeric proteins can be prepared by transfecting DNA encoding these chimeric receptor proteins to suitable host cells and culturing these cells under suitable conditions.

It is extremely practical if, next to the information for the expression of the steroid receptor, also the host cell is transformed or transfected with a vector which carries the information for a reporter molecule. Such a vector coding for a reporter molecule is characterized by having a promoter sequence containing one or more hormone responsive elements (HRE) functionally linked to an operative reporter gene. Such a HRE is the DNA target of the activated steroid receptor and, as a consequence, it enhances the transcription of the DNA coding for the reporter molecule. In *in vivo* settings of steroid receptors the reporter molecule comprises the cellular response to the stimulation of the ligand. However, it is possible *in vitro* to combine the ligand-binding domain of a

receptor to the DNA binding domain and transcription  
activating domain of other steroid receptors, thereby enabling  
the use of other HRE and reporter molecule systems. One such a  
system is established by a HRE presented in the MMTV-LTR  
5 (mouse mammary tumor virus long terminal repeat sequence in  
connection with a reporter molecule like the firefly  
luciferase gene or the bacterial gene for CAT (chloramphenicol  
transferase). Other HRE's which can be used are the rat  
oxytocin promotor, the retinoic acid responsive element, the  
10 thyroid hormone responsive element, the estrogen responsive  
element and also synthetic responsive elements have been  
described (for instance in Fuller, *ibid.* page 3096). As  
reporter molecules next to CAT and luciferase  $\beta$ -galactosidase  
can be used.

Steroid hormone receptors and chimeric receptors according  
to the present invention can be used for the *in vitro*  
identification of novel ligands or hormonal analogs. For this  
purpose binding studies can be performed with cells  
20 transformed with DNA according to the invention or an  
expression vector comprising DNA according to the invention,  
said cells expressing the steroid receptors or chimeric  
receptors according to the invention.

The novel steroid hormone receptor and chimeric receptors  
according to the invention as well as the ligand-binding  
domain of the invention, can be used in an assay for the  
identification of functional ligands or hormone analogs for  
30 the nuclear receptors.

Thus, the present invention provides for a method for  
identifying functional ligands for the steroid receptors and  
chimeric receptors according to the invention, said method  
comprising the steps of

- a) introducing into a suitable host cell 1) DNA or an expression vector according to the invention, and 2) a suitable reporter gene functionally linked to an operative hormone response element, said HRE being able to be activated by the DNA-binding domain of the receptor protein encoded by said DNA;
- b) bringing the host cell from step a) into contact with potential ligands which will possibly bind to the ligand-binding domain of the receptor protein encoded by said DNA from step a);
- c) monitoring the expression of the receptor protein encoded by said reporter gene of step a).

If expression of the reporter gene is induced with respect to basic expression (without ligand), the functional ligand can be considered as an agonist; if expression of the reporter gene remains unchanged or is reduced with respect to basic expression, the functional ligand can be a suitable (partial) antagonist.

For performing such kind of investigations host cells which have been transformed or transfected with both a vector encoding a functional steroid receptor and a vector having the information for a hormone responsive element and a connected reporter molecule are cultured in a suitable medium. After addition of a suitable ligand, which will activate the receptor the production of the reporter molecule will be enhanced, which production simply can be determined by assays having a sensitivity for the reporter molecule. See for instance WO-A-8803168. Assays with known steroid receptors have been described (for instance S. Tsai et al., Cell 57, 443, 1989; M. Meyer et al., Cell 57, 433, 1989).

## Legends to the figures

### **Figure 1.**

Northern analysis of the novel estrogen receptor (ER $\beta$ ). Two  
5 different multiple tissue Northern blots (Clontech) were  
hybridised with a specific probe for ER $\beta$  (see examples).  
Indicated are the human tissues the RNA originated from and  
the position of the size markers in kilobases (kb).

### **Figure 2.**

10 Histogram showing the 3- to 4-fold stimulatory effect of  
17 $\beta$ -estradiol, estriol and estrone on the luciferase activity  
mediated by ER $\beta$ . An expression vector encoding ER $\beta$  was  
transiently transfected into CHO cells together with a  
15 reporter construct containing the rat oxytocin promoter in  
front of the firefly luciferase encoding sequence (see  
examples).

### **Figure 3.**

20 Effect of 17 $\beta$ -estradiol (E2) alone or in combination with  
the anti-estrogen ICI-164384 (ICI) on ER $\alpha$  and ER $\beta$ . Expression  
constructs for ER $\alpha$  (the classical ER) and ER $\beta$  were transiently  
transfected into CHO cells together with the rat oxytocin  
promoter-luciferase reporter construct described in the  
25 examples. Luciferase activities were determined in triplicate  
and normalised for transfection efficiency by measuring  $\beta$ -  
galactosidase in the same lysate.

**Figure 4.**

Expression of ER $\alpha$  and ER $\beta$  in a number of cell lines determined by RT-PCR analysis (see examples). The cell lines used were derived from different tissues/cell types:

5 endometrium (ECC1, Ishikawa, HEC-1A, RL95-2); osteosarcoma (SAOS-2, U2-OS, HOS, MG63); breast tumours (MCF-7, T47D), endothelium (HUV-EC-C, BAEC-1); smooth muscle (HISM, PAC-1, A7R5, A10, RASMC, CavaSMC); liver (HepG2); colon (CaCo2); and vagina (Hs-760T, SW-954).

10 All cell lines were human except for PAC-1, A7R5, A10 and RASMC which are of rat origin, BAEC-1 which is of bovine origin and CavaSMC which is of guinea pig origin.

**Figure 5.**

15 Transactivation assay using stably transfected CHO cell lines expressing ER $\alpha$  or ER $\beta$  together with the rat oxytocin-luciferase estrogen-responsive reporter (see examples for details). Hormone-dependent transactivation curves were determined for 17 $\beta$ -estradiol and for Org4094. For the ER

20 antagonist raloxifen, cells were treated with  $2 \times 10^{-10}$  mol/L 17 $\beta$ -estradiol together with increasing concentrations of raloxifen. Maximal values of the responses were arbitrarily set at 100%.

**Examples**

**A. Molecular cloning of the novel estrogen receptor.**

Two degenerate oligonucleotides containing inosines (I)

30 were based on conserved regions of the DNA-binding domains and the ligand-binding domains of the human steroid hormone receptors.

Primer #1:

5'-GGIGA(C/T)GA(A/G)GC(A/T)TCIGGITG(C/T)CA(C/T)TA(C/T)GG-3'  
(SEQ ID NO:7).

Primer #2:

5'-AAGCCTGG(C/G)A(C/T)IC(G/T)(C/T)TTIGCCCAI(C/T)TIAT-3' SEQ  
ID NO:8).

As template, cDNA from human EBV-stimulated PBLs  
(peripheral blood leukocytes) was used. One microgram of total  
RNA was reverse transcribed in a 20 µl reaction containing 50  
mM KCl, 10 mM Tris-HCl pH 8.3, 4 mM MgCl<sub>2</sub>, 1 mM dNTPs  
(Pharmacia), 100 pmol random hexanucleotides (Pharmacia), 30  
Units RNase inhibitor (Pharmacia) and 200 Units M-MLV Reverse  
transcriptase (Gibco BRL). Reaction mixtures were incubated at  
37°C for 30 minutes and heat-inactivated at 100°C for 5  
minutes. The cDNA obtained was used in a 100 µl PCR reaction  
containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl<sub>2</sub>,  
0.001% gelatin (w/v), 3% DMSO, 1 microgram of primer #1 and  
primer #2 and 2.5 Units of Amplitaq DNA polymerase (Perkin  
Elmer). PCR reactions were performed in the Perkin Elmer 9600  
thermal cycler. The initial denaturation (4 minutes at 94°C)  
was followed by 35 cycles with the following conditions: 30  
sec. 94°C, 30 sec. 45°C, 1 minute 72°C and after 7 minutes at  
72°C the reactions were stored at 4°C. Aliquots of these  
reactions were analysed on a 1.5% agarose gel. Fragments of  
interest were cut out of the gel, reamplified using identical  
PCR-conditions and purified using Qiaex II (Qiagen). Fragments  
were cloned in the pCRII vector and transformed into bacteria  
using the TA-cloning kit (Invitrogen). Plasmid DNA was  
isolated for nucleotide sequence analysis using the Qiagen  
plasmid midi protocol (Qiagen). Nucleotide sequence analysis  
was performed with the ALF automatic sequencer (Pharmacia)  
using a T7 DNA sequencing kit (Pharmacia) with vector-specific  
or fragment-specific primers.

One cloned fragment corresponded to a novel estrogen receptor (ER) which is closely related to the classical estrogen receptor. Part of the cloned novel estrogen receptor fragment (nucleotides 466 to 797 in SEQ ID 1) was amplified by PCR using oligonucleotide #3 TGTACGAAGTGGGAATGGTGA (SEQ ID NO:9) and oligonucleotide #2 and used as a probe to screen a human testis cDNA library in  $\lambda$ gt11 (Clontech #HL1010b). Recombinant phages were plated (using Y1090 bacteria grown in LB medium supplemented with 0.2% maltose) at a density of 40.000 pfu (plaque-forming units) per 135 mm dish and replica filters (Hybond-N, Amersham) were made as described by the supplier. Filters were prehybridised in a solution containing 0.5 M phosphate buffer (pH 7.5) and 7% SDS at 65°C for at least 30 minutes. DNA probes were purified with Qiaex II (Qiagen), <sup>32</sup>P-labeled with a Decaprime kit (Ambion) and added to the prehybridisation solution. Filters were hybridised at 65°C overnight and then washed in 0.5 X SSC/0.1% SDS at 65°C. Two positive plaques were identified and could be shown to be identical. These clones were purified by rescreening one more time. A PCR reaction on the phage eluates with the  $\lambda$ gt11-specific primers #4: 5'-TTGACACCAGACCAACTGGTAATG-3' (SEQ ID NO:10) and #5: 5'-GGTGGCGACGACTCCTGGAGCCCG-3' (SEQ ID NO:11) yielded a fragment of 1700 basepairs on both clones. Subsequent PCR reactions using combinations of a gene-specific primer #6: 5'-GTACACTGATTTGTAGCTGGAC-3' (SEQ ID NO:12) with the  $\lambda$ gt11 primer #4 and gene-specific primer #7: 5'-CCATGATGATGTCCTGACC-3' (SEQ ID NO:13) with  $\lambda$ gt11 primer #5 yielded fragments of approximately 450 bp and 1000 bp, respectively, which were cloned in the pCRII vector and used for nucleotide sequence analysis. The conditions for these PCR reactions were as described above except for the primer concentrations (200 ng of each primer) and the annealing temperature (60°C). Since in the cDNA clone the



homology with the ER is lost abruptly at a site which corresponds to the exon 7/exon 8 boundary in the ER (between nucleotides 1247 and 1248 in SEQ ID NO:1), it was suggested that this sequence corresponds to intron 7 of the novel ER gene. For verification of the nucleotide sequences of this cDNA clone, a 1200 bp fragment was generated on the cDNA clone with  $\lambda$ gt11 primer #4 with a gene-specific primer #8 corresponding to the 3' end of exon 7: 5'-TCGCATGCCTGACGTGGGAC-3' (SEQ ID NO:14) using the proofreading *Pfu* polymerase (Stratagene). This fragment was also cloned in the pCRII vector and completely sequenced and was shown to be identical to the sequences obtained earlier.

To obtain nucleotide sequences of the novel ER downstream of exon 7, a degenerate oligonucleotide based on the AF-2 region of the classical ER (#9: 5'-GGC(C/G)TCCAGCATCTCCAG(C/G)A(A/G)CAG-3'; SEQ ID NO:15) was used together with the gene-specific oligonucleotide #10: 5'-GGAAGCTGGCTCACTTGCTG-3' (SEQ ID NO:16) using testis cDNA as template (Marathon ready testis cDNA, Clontech Cat #7414-1). A specific 220 bp fragment corresponding to nucleotides 1112 to 1332 in SEQ ID No. 1 was cloned and sequenced. Nucleotides 1112 to 1247 were identical to the corresponding sequence of the cDNA clone. The sequence downstream thereof is highly homologous with the corresponding region in the classical ER. In order to obtain sequences of the novel ER downstream of the AF-2 region, RACE (rapid amplification of cDNA ends) PCR reactions were performed using the Marathon-ready testis cDNA (Clontech) as template. The initial PCR was performed using oligonucleotide #11: 5'-TCTTGTCTGGACAGGGATG-3' (SEQ ID NO:17) in combination with the AP1 primer provided in the kit. A nested PCR was performed on an aliquot of this reaction using oligonucleotide #10 (SEQ ID NO:16) in combination with the oligo dT primer provided in the kit. Subsequently, an aliquot of this reaction was used in a nested PCR using

oligonucleotide #12: 5'-GCATGGAACATCTGCTCAAC-3' (SEQ ID NO:18) in combination with the oligo dT primer. Nucleotide sequence analysis of a specific fragment that was obtained (corresponding to nucleotides 1256 to 1431 in SEQ ID NO 1) revealed a sequence encoding the carboxyterminus of the novel ER ligand-binding domain, including an F-domain and a translational stop codon and part of the 3' untranslated sequence which is not included in SEQ ID NO:1. The deduced amino acid sequence is shown in SEQ ID NO:5.

In order to investigate the possibility that the novel estrogen receptor had additional, upstream translation-initiation codons, RACE-PCR experiments were performed using Marathon-ready testis cDNA (Clontech Cat. # 7414-1). First a PCR was performed using oligonucleotide SEQ ID NO:12 (antisense corresponding to nucleotides 416-395 in SEQ ID NO:1) and AP-1 (provided in the kit). A nested PCR was then performed using oligonucleotide having SEQ ID NO:27 (antisense corresponding to nucleotides 254-231 in SEQ ID NO:1) with AP-2 (provided in the kit). From the smear that was obtained, the region corresponding to fragments larger than 300 basepairs was cut out, purified using the GenecleanII kit (Bio101) and cloned using the TA-cloning kit (Clontech). Colonies were screened by PCR using gene-specific primers: SEQ ID NO:22 and SEQ ID NO:28. The clone containing the largest insert was sequenced. The nucleotide sequence corresponds to nucleotides 1 to 490 in SEQ ID NO:24. It is clear from this sequence that the first in-frame upstream translation initiation codon is present at position 77-79 in SEQ ID NO:24. Upstream of this translational startcodon an in-frame stop-codon is present (11-13 in SEQ ID NO:24). Consequently, the reading frame of the novel estrogen receptor is 530 amino acids (shown in SEQ ID NO:25) and has a calculated molecular mass of 59.234 kD.

To confirm the nucleotide sequences obtained by 5' RACE, human genomic clones were obtained and analysed. A human genomic library in  $\lambda$ EMBL3 (Clontech HL1067J) was screened with a probe corresponding to nucleotides 1 to 416 in SEQ ID NO:1. A strongly hybridizing clone was plaque-purified and DNA was isolated using standard protocols (Sambrook et al, 1989). The DNA was digested with several restriction enzymes, electrophoresed on agarose gel and blotted onto Nylon filters. Hybridisation of the blot with a probe corresponding to the above-mentioned RACE fragment (nucleotides 1-490 in SEQ ID NO:24) revealed a hybridizing Sau3A fragment of approximately 800 basepairs. This fragment was cloned into the BamH1 site of pGEM3Z and sequenced. The nucleotide sequence contained one base difference which is probably a PCR-induced point mutation in the RACE fragment. Nucleotide 172 was a G residue in the 5'RACE fragment, but an A residue in several independent genomic subclones.

***B. Identification of two splice variants of the novel estrogen receptor.***

Rescreening of the testis cDNA library with a probe corresponding to nucleotides 918 to 1246 in SEQ ID No. 1 yielded two hybridizing clones, the 3' end of which were amplified by PCR (gene-specific primer #10: 5'-GGAAGCTGGCTCACTTGCTG-3' (SEQ ID NO:16) together with primer #4, SEQ ID NO:10), cloned and sequenced. One clone was shown to contain an alternative exon 8 (exon 8B) of the novel ER. In SEQ ID No. 2 the protein encoding part and the stopcodon of this splice variant are presented. As a consequence of the introduction of this exon through an alternative splicing reaction, the reading frame encoding the novel ER is immediately terminated, thereby creating a truncation of the carboxyterminus of the novel ER (SEQ ID NO:6).

Screening of a human thymus cDNA library (Clontech HL1074a) with the probe corresponding to nucleotides 918 to 1246 in SEQ ID No. 1, revealed another splice variant. The 3' end of one hybridizing clone was amplified using primer #10 (SEQ ID NO:16) with the  $\lambda$ gt10-specific primer #13 5'-AGCAAGTTCAGCCTGTTAAGT-3' (SEQ ID NO:19), cloned and sequenced. The obtained nucleotide sequence upstream of the exon 7/exon 8 boundary was identical to the clones identified earlier. However, an alternative exon 8 (exon 8C) was present at the 3' end encoding two C-terminal amino acids followed by a stop-codon. The nucleotide sequence of the protein-encoding part of this splice variant is shown in SEQ ID NO:20, the corresponding protein sequence is SEQ ID NO:21.

These two variants of the novel estrogen receptor do not contain the AF-2 region and therefore probably lack the ability to modulate transcription of target genes in a ligand-dependent fashion. However, the variants potentially could interfere with the functioning of the wild-type classical ER and/or the wild-type novel ER, either by heterodimerization or by occupying estrogen response elements or by interactions with other transcription factors. A mutant of the classical ER (ER1-530) has been described which closely resembles the two variants of the novel estrogen receptor described above. ER1-530 has been shown to behave as a dominant-negative receptor i.e. it can modulate the intracellular activity of the wild type ER (Ince et al, J. Biol. Chem. 268, 14026-14032, 1993).

### **C. Northern blot analysis.**

Human multiple tissue Northern blots (MTN-blots) were purchased from Clontech and prehybridized for at least 1 hour at 65°C in 0.5 M phosphate buffer pH 7.5 with 7% SDS. The DNA fragment that was used as a probe (corresponding to

nucleotides 466 to 797 in SEQ ID No. 1) was <sup>32</sup>P-labeled using a labelling kit (Ambion), denatured by boiling and added to the prehybridisation solution. Washing conditions were: 3X SSC at room temperature, followed by 3 X SSC at 65°C, and finally 1 X SSC at 65°C. The filters were then exposed to X-ray films for one week. Two transcripts of approximately 8 kb and 10 kb were detected in thymus, spleen, ovary and testis. In addition, a 1.3 kb transcript was detected in testis.

***D. RT-PCR analysis of expression of ER $\alpha$  and ER $\beta$  in cell lines.***

RNA was isolated from a number of human and animal cell lines using RNazol B (Cinna/Biotechx). cDNA was made using 2.5 microgram of total RNA using the Superscript II kit (BRL) following the manufacturers instructions. A portion of the cDNA was used for specific PCR amplifications of fragments corresponding either to mRNA encoding the ER or to the novel estrogen receptor. (It should be emphasized that the primers used are based on human and rat sequences, whereas some of the cell lines were not rat or human, see legend of Figure 4). Primers used were for ER $\alpha$ : sense 5'-GATGGGCTTACTGACCAACC-3' and antisense 5'-AGATGCTCCATGCCTTTG-3' generating a 548 base pair fragment corresponding to part of the LBD. For ER $\beta$ : sense 5'- TTCACCGAGGCCTCCATGATG-3' and antisense 5'- CAGATGTTCCATGCCCTTGTT-3' generating a 565 base pair fragment corresponding to part of the LBD. The PCR samples were analysed on agarose which were blotted onto Nylon membranes. These blots were hybridised with <sup>32</sup>P-labeled PCR fragments generated with the above-mentioned primers on ER $\alpha$  and ER $\beta$  plasmid DNA using standard experimental procedures (Sambrook et al, 1989).

***E. Ligand-dependent transcription activation by the novel estrogen receptor protein.***

Cell culture

5 Chinese Hamster Ovary (CHO K1) cells were obtained from ATCC (CCL61) and maintained at 37°C in a humidified atmosphere (5% CO<sub>2</sub>) as a monolayer culture in fenolred-free M505 medium. The latter medium consists of a mixture (1:1) of Dulbecco's Modified Eagle's Medium (DMEM, Gibco 074-200) and Nutrient  
10 Medium F12 (Ham's F12, Gibco 074-1700) supplemented with 2.5 mg/ml sodium carbonate (Baker), 55 µg/ml sodium pyruvate (Fluka), 2.3 µg/ml β-mercaptoethanol (Baker), 1.2 µg/ml ethanolamine (Baker), 360 µg/ml L-glutamine (Merck), 0.45 µg/ml sodium selenite (Fluka), 62.5 µg/ml penicillin  
15 (Mycopharm), 62.5 µg/ml streptomycin (Serva), and 5% charcoal-treated bovine calf serum (Hyclone).

Recombinant vectors

The ERβ-encoding sequence as presented in SEQ ID No. 1 was amplified by PCR using oligonucleotides 5'-  
20 CTTGGATCCATAGCCCTGCTGTGATGAATTACAG-3' (SEQ ID NO:22 underlined is the translation initiation codon) in combination with 5'-GATGGATCCTCACCTCAGGGCCAGGCGTCACTG-3' (SEQ ID NO:23) (underlined is the translation stopcodon, antisense). The resulting BamH1 fragment (approximately 1450 base pairs) were  
25 then cloned in the mammalian cell expression vector pNGV1 (Genbank accession No. X99274).

An expression construct encoding the ERβ reading frame as presented in SEQ ID NO:24 was made by replacing a BamH1-MscI fragment (nucleotides 1-81 in SEQ ID No. 1) by a BamH1-MscI  
30 fragment corresponding to nucleotides 77-316 in SEQ ID No. 24. The latter fragment was made by PCR with SEQ ID NO:26 in

combination with SEQ ID NO:28 using the above mentioned 5' RACE fragment.

The reporter vector was based on the rat oxytocin gene regulatory region (position -363/+16 as a HindIII/ MboI fragment; R.Ivell, and D.Richter, Proc.Natl.Acad.Sci.USA 81, 2006-2010, 1984) linked to the firefly luciferase encoding sequence; the regulatory region of the oxytocin gene was shown to possess functional estrogen hormone response elements *in vitro* for both the rat (R.Adan *et al*, Biochem.Biophys.Res.Comm. 175, 117-122, 1991) and the human (S.Richard, and H.Zingg, J.Biol.Chem. 265, 6098-6103, 1990).

#### Transient transfection

1 x 10<sup>5</sup> CHO cells were seeded in 6-wells Nunclon tissue culture plates and DNA was introduced by use of lipofectin (Gibco BRL). Hereto, the DNA (1 µg of both receptor and reporter vector in 250 µL Optimem, Gibco BRL) was mixed with an equal volume of lipofectin reagent (7 µL in 250 µL Optimem, Gibco) and allowed to stand at room temperature for 15 min. After washing the cells twice with serum-free medium (M505) new medium (500 µL Optimem, Gibco) was added to the cells followed by the dropwise addition of the DNA-lipofectin mixture. After incubation for a 5 hour period at 37°C cells were washed twice with fenolred-free M505 + 5% charcoal-treated bovine calf serum and incubated overnight at 37°C. After 24 hours hormones were added to the medium (10<sup>-7</sup> mol/L). Cell extracts were made 48 hours posttransfection by the addition of 200 µL lysisbuffer (0.1 M phosphate buffer pH7.8, 0.2% Triton X-100). After incubation for 5 min at 37°C the cell suspension was centrifuged (Eppendorf centrifuge, 5 min) and 20 µL sample was added to 50 µL luciferase assay reagent (Promega). Light emission was measured in a luminometer (Berthold Biolumat) for 10 sec at 562 nm.

Stable transfection of the novel estrogen receptor.

The expression plasmid encoding full-length ER $\beta$ 1-530 (see above) was stably transfected in CHO K1 cells as previously described (Theunissen et al., J. Biol. Chem. 268, 9035-9040, 1993). Single cell clones that were obtained this way were screened by transient transfection of the reporter plasmid (rat oxytocin-luciferase) as described above. Selected clones were used for a second stable transfection of the rat oxytocin-luciferase reporter plasmid together with the plasmid pDR2A which contains a hygromycine resistance gene for selection. Single cell clones obtained were tested for a response to 17 $\beta$ -estradiol. Subsequently, a selected single cell clone was used for transactivation studies. Briefly, cells were seeded in 96-wells at ( $1.6 \times 10^4$  cells per well). After 24 hours different concentrations of hormone were diluted in medium and added to the wells. For antagonistic experiments,  $2 \times 10^{-10}$  M. 17 $\beta$ -estradiol was added to each well and different concentrations of antagonists were added. Cells were washed once with PBS after a 24 hour incubation and then lysed by the addition of 40 microliter lysis buffer (see above). Luciferase reagent was added (50 microliter) to each well and light emission was measured using the Topcount (Packard).

Results.

A comparison of the two expression constructs (SEQ ID NO:1 and SEQ ID NO:24) in transient transfections in CHO cells showed identical transactivation in response to a number of agonists and antagonists. CHO cells transiently transfected with ER $\beta$  expression vector and a reporter plasmid showed a 3 to 4 fold increase in luciferase activity in response to 17 $\beta$ -estradiol as compared to untreated cells (see Figure 2). A similar transactivation was obtained upon treatment with estriol and estrone. The results indicate not only that the



novel ER (ER $\beta$ ) can bind estrogen hormones but also that the ligand-activated receptor can bind to the estrogen-response elements (EREs) within the rat oxytocin promoter and activate transcription of the luciferase reporter gene. Figure 3 shows that in an independent similar experiment  $10^{-9}$  mol/L 17 $\beta$ -estradiol gave an 18-fold stimulation with ER $\alpha$  and a 7-fold stimulation with ER $\beta$ . In addition, the antiestrogen ICI-164384 was shown to be an antagonist for both ER $\alpha$  and ER $\beta$  when activated with 17 $\beta$ -estradiol, whereas the antagonist alone had no effect. In this experiment 0.25  $\mu$ g  $\beta$ -galactosidase vector was co-transfected in order to normalize for differences in transfection efficiency.

Transactivation studies performed on stably transfected ER $\alpha$  and ER $\beta$  cell lines gave similar absolute luciferase values. The curves for 17 $\beta$ -estradiol are very similar and show that half-maximal transactivation is reached with lower concentrations of hormone on ER $\alpha$  as compared to ER $\beta$  (Figure 5). For Org4094 this is also the case however, the effect observed is much more pronounced. The curves for raloxifen show that the potency of this antagonist to block transactivation on ER $\alpha$  is greater compared to its potency to block ER $\beta$  transactivation.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

(i) APPLICANT:

- (A) NAME: Akzo nobel n.v.
- (B) STREET: Velperweg 76
- (C) CITY: Arnhem
- (E) COUNTRY: The Netherlands
- (F) POSTAL CODE (ZIP): 6824 BM
- (G) TELEPHONE: 0412-666379
- (H) TELEFAX: 0412-650592
- (I) TELEX: 37503 akpha nl

10

15

(ii) TITLE OF INVENTION: Novel estrogen receptor

(iii) NUMBER OF SEQUENCES: 28

20

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

25

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

30

- (A) LENGTH: 1434 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:



ACCGATGCTT TGGTTTGGGT GATTGCCAAG AGCGGCATCT CCTCCCAGCA GCAATCCATG 1200  
CGCCTGGCTA ACCTCCTGAT GTCCTGTCC CACGTCAGGC ATGCGAGTAA CAAGGGCATG 1260  
5 GAACATCTGC TCAACATGAA GTGCAAAAAT GTGGTCCCAG TGTATGACCT GCTGCTGGAG 1320  
ATGCTGAATG CCCACGTGCT TCGCGGGTGC AAGTCCTCCA TCACGGGGTC CGAGTGCAGC 1380  
CCGGCAGAGG ACAGTAAAAG CAAAGAGGGC TCCCAGAACC CACAGTCTCA GTGA 1434

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1251 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

25 ATGAATTACA GCATTCCCAG CAATGTCAC TAACTTGAAG GTGGGCCTGG TCGGCAGACC 60  
ACAAGCCCAA ATGTGTTGTG GCCAACACCT GGGCACCTTT CTCCTTTAGT GGTCCATCGC 120  
CAGTTATCAC ATCTGTATGC GGAACCTCAA AAGAGTCCCT GGTGTGAAGC AAGATCGCTA 180  
30 GAACACACCT TACCTGTAAA CAGAGAGACA CTGAAAAGGA AGGTTAGTGG GAACCGTTGC 240  
GCCAGCCCTG TTACTGGTCC AGGTTCAAAG AGGGATGCTC ACTTCTGCGC TGTCTGCAGC 300  
35 GATTACGCAT CGGGATATCA CTATGGAGTC TGGTCGTGTG AAGGATGTAA GGCCTTTTTT 360  
AAAAGAAGCA TTCAAGGACA TAATGATTAT ATTTGTCCAG CTACAAATCA GTGTACAATC 420  
GATAAAAACC GGCACAAGAG CTGCCAGGCC TGCCGACTTC GGAAGTGTTA CGAAGTGGGA 480

ATGGTGAAGT GTGGCTCCCG GAGAGAGAGA TGTGGGTACC GCCTTGTGCG GAGACAGAGA 540  
AGTGCCGACG AGCAGCTGCA CTGTGCCGGC AAGGCCAAGA GAAGTGGCGG CCACGCGCCC 600  
5 CGAGTGCGGG AGCTGCTGCT GGACGCCCTG AGCCCCGAGC AGCTAGTGCT CACCCTCCTG 660  
GAGGCTGAGC CGCCCCATGT GCTGATCAGC CGCCCCAGTG CGCCCTTCAC CGAGGCCTCC 720  
10 ATGATGATGT CCCTGACCAA GTTGCCGAC AAGGAGTTGG TACACATGAT CAGCTGGGCC 780  
AAGAAGATTC CCGGCTTTGT GGAGCTCAGC CTGTTCGACC AAGTGCGGCT CTTGGAGAGC 840  
TGTTGGATGG AGGTGTTAAT GATGGGGCTG ATGTGGCGCT CAATTGACCA CCCC GGCAAG 900  
15 CTCATCTTTG CTCCAGATCT TGTTCTGGAC AGGGATGAGG GGAAATGCGT AGAAGGAATT 960  
CTGGAAATCT TTGACATGCT CCTGGCAACT ACTTCAAGGT TTCGAGAGTT AAAACTCCAA 1020  
20 CACAAAGAAT ATCTCTGTGT CAAGGCCATG ATCCTGCTCA ATTCCAGTAT GTACCCTCTG 1080  
GTCACAGCGA CCCAGGATGC TGACAGCAGC CGGAAGCTGG CTCACTTGCT GAACGCCGTG 1140  
ACCGATGCTT TGGTTTGGGT GATTGCCAAG AGCGGCATCT CCTCCCAGCA GCAATCCATG 1200  
25 CGCCTGGCTA ACCTCCTGAT GCTCCTGTCC CACGTCAGGC ATGCGAGGTG A 1251

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 66 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Cys Ala Val Cys Ser Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp  
1 5 10 15  
Ser Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His  
20 25 30  
Asn Asp Tyr Ile Cys Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn  
35 40 45  
Arg Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val  
50 55 60  
Gly Met  
65

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Leu Val Leu Thr Leu Leu Glu Ala Glu Pro Pro His Val Leu Ile Ser  
1 5 10 15  
Arg Pro Ser Ala Pro Phe Thr Glu Ala Ser Met Met Met Ser Leu Thr  
20 25 30  
Lys Leu Ala Asp Lys Glu Leu Val His Met Ile Ser Trp Ala Lys Lys  
35 40 45

Ile Pro Gly Phe Val Glu Leu Ser Leu Phe Asp Gln Val Arg Leu Leu  
50 55 60

5 Glu Ser Cys Trp Met Glu Val Leu Met Met Gly Leu Met Trp Arg Ser  
65 70 75 80

Ile Asp His Pro Gly Lys Leu Ile Phe Ala Pro Asp Leu Val Leu Asp  
85 90 95

10 Arg Asp Glu Gly Lys Cys Val Glu Gly Ile Leu Glu Ile Phe Asp Met  
100 105 110

15 Leu Leu Ala Thr Thr Ser Arg Phe Arg Glu Leu Lys Leu Gln His Lys  
115 120 125

Glu Tyr Leu Cys Val Lys Ala Met Ile Leu Leu Asn Ser Ser Met Tyr  
130 135 140

20 Pro Leu Val Thr Ala Thr Gln Asp Ala Asp Ser Ser Arg Lys Leu Ala  
145 150 155 160

His Leu Leu Asn Ala Val Thr Asp Ala Leu Val Trp Val Ile Ala Lys  
165 170 175

25 Ser Gly Ile Ser Ser Gln Gln Gln Ser Met Arg Leu Ala Asn Leu Leu  
180 185 190

30 Met Leu Leu Ser His Val Arg His Ala Ser Asn Lys Gly Met Glu His  
195 200 205

Leu Leu Asn Met Lys Cys Lys Asn Val Val Pro Val Tyr Asp Leu Leu  
210 215 220

35 Leu Glu Met Leu Asn Ala His Val Leu  
225 230

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 477 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Asn Tyr Ser Ile Pro Ser Asn Val Thr Asn Leu Glu Gly Gly Pro  
1 5 10 15

Gly Arg Gln Thr Thr Ser Pro Asn Val Leu Trp Pro Thr Pro Gly His  
20 25 30

Leu Ser Pro Leu Val Val His Arg Gln Leu Ser His Leu Tyr Ala Glu  
35 40 45

Pro Gln Lys Ser Pro Trp Cys Glu Ala Arg Ser Leu Glu His Thr Leu  
50 55 60

Pro Val Asn Arg Glu Thr Leu Lys Arg Lys Val Ser Gly Asn Arg Cys  
65 70 75 80

Ala Ser Pro Val Thr Gly Pro Gly Ser Lys Arg Asp Ala His Phe Cys  
85 90 95

Ala Val Cys Ser Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser  
100 105 110

Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn  
115 120 125

Asp Tyr Ile Cys Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn Arg  
130 135 140



Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val Gly  
145 150 155 160

Met Val Lys Cys Gly Ser Arg Arg Glu Arg Cys Gly Tyr Arg Leu Val  
165 170 175

Arg Arg Gln Arg Ser Ala Asp Glu Gln Leu His Cys Ala Gly Lys Ala  
180 185 190

Lys Arg Ser Gly Gly His Ala Pro Arg Val Arg Glu Leu Leu Leu Asp  
195 200 205

Ala Leu Ser Pro Glu Gln Leu Val Leu Thr Leu Leu Glu Ala Glu Pro  
210 215 220

Pro His Val Leu Ile Ser Arg Pro Ser Ala Pro Phe Thr Glu Ala Ser  
225 230 235 240

Met Met Met Ser Leu Thr Lys Leu Ala Asp Lys Glu Leu Val His Met  
245 250 255

Ile Ser Trp Ala Lys Lys Ile Pro Gly Phe Val Glu Leu Ser Leu Phe  
260 265 270

Asp Gln Val Arg Leu Leu Glu Ser Cys Trp Met Glu Val Leu Met Met  
275 280 285

Gly Leu Met Trp Arg Ser Ile Asp His Pro Gly Lys Leu Ile Phe Ala  
290 295 300

Pro Asp Leu Val Leu Asp Arg Asp Glu Gly Lys Cys Val Glu Gly Ile  
305 310 315 320

Leu Glu Ile Phe Asp Met Leu Leu Ala Thr Thr Ser Arg Phe Arg Glu  
325 330 335

Leu Lys Leu Gln His Lys Glu Tyr Leu Cys Val Lys Ala Met Ile Leu  
340 345 350

Leu Asn Ser Ser Met Tyr Pro Leu Val Thr Ala Thr Gln Asp Ala Asp  
355 360 365

Ser Ser Arg Lys Leu Ala His Leu Leu Asn Ala Val Thr Asp Ala Leu  
370 375 380

Val Trp Val Ile Ala Lys Ser Gly Ile Ser Ser Gln Gln Gln Ser Met  
385 390 395 400

Arg Leu Ala Asn Leu Leu Met Leu Leu Ser His Val Arg His Ala Ser  
405 410 415

Asn Lys Gly Met Glu His Leu Leu Asn Met Lys Cys Lys Asn Val Val  
420 425 430

Pro Val Tyr Asp Leu Leu Leu Glu Met Leu Asn Ala His Val Leu Arg  
435 440 445

Gly Cys Lys Ser Ser Ile Thr Gly Ser Glu Cys Ser Pro Ala Glu Asp  
450 455 460

Ser Lys Ser Lys Glu Gly Ser Gln Asn Pro Gln Ser Gln  
465 470 475

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 416 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Met Asn Tyr Ser Ile Pro Ser Asn Val Thr Asn Leu Glu Gly Gly Pro

1	5	10	15
Gly Arg Gln Thr Thr Ser Pro Asn Val Leu Trp Pro Thr Pro Gly His	20	25	30
5			
Leu Ser Pro Leu Val Val His Arg Gln Leu Ser His Leu Tyr Ala Glu	35	40	45
10			
Pro Gln Lys Ser Pro Trp Cys Glu Ala Arg Ser Leu Glu His Thr Leu	50	55	60
15			
Pro Val Asn Arg Glu Thr Leu Lys Arg Lys Val Ser Gly Asn Arg Cys	65	70	75
20			
Ala Ser Pro Val Thr Gly Pro Gly Ser Lys Arg Asp Ala His Phe Cys	85	90	95
25			
Ala Val Cys Ser Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser	100	105	110
30			
Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn	115	120	125
35			
Asp Tyr Ile Cys Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn Arg	130	135	140
40			
Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val Gly	145	150	155
45			
Met Val Lys Cys Gly Ser Arg Arg Glu Arg Cys Gly Tyr Arg Leu Val	165	170	175
50			
Arg Arg Gln Arg Ser Ala Asp Glu Gln Leu His Cys Ala Gly Lys Ala	180	185	190
55			
Lys Arg Ser Gly Gly His Ala Pro Arg Val Arg Glu Leu Leu Asp	195	200	205
60			
Ala Leu Ser Pro Glu Gln Leu Val Leu Thr Leu Leu Glu Ala Glu Pro			

210

215

220

Pro His Val Leu Ile Ser Arg Pro Ser Ala Pro Phe Thr Glu Ala Ser  
225 230 235 240

Met Met Met Ser Leu Thr Lys Leu Ala Asp Lys Glu Leu Val His Met  
245 250 255

Ile Ser Trp Ala Lys Lys Ile Pro Gly Phe Val Glu Leu Ser Leu Phe  
260 265 270

Asp Gln Val Arg Leu Leu Glu Ser Cys Trp Met Glu Val Leu Met Met  
275 280 285

Gly Leu Met Trp Arg Ser Ile Asp His Pro Gly Lys Leu Ile Phe Ala  
290 295 300

Pro Asp Leu Val Leu Asp Arg Asp Glu Gly Lys Cys Val Glu Gly Ile  
305 310 315 320

Leu Glu Ile Phe Asp Met Leu Leu Ala Thr Thr Ser Arg Phe Arg Glu  
325 330 335

Leu Lys Leu Gln His Lys Glu Tyr Leu Cys Val Lys Ala Met Ile Leu  
340 345 350

Leu Asn Ser Ser Met Tyr Pro Leu Val Thr Ala Thr Gln Asp Ala Asp  
355 360 365

Ser Ser Arg Lys Leu Ala His Leu Leu Asn Ala Val Thr Asp Ala Leu  
370 375 380

Val Trp Val Ile Ala Lys Ser Gly Ile Ser Ser Gln Gln Gln Ser Met  
385 390 395 400

Arg Leu Ala Asn Leu Leu Met Leu Leu Ser His Val Arg His Ala Arg  
405 410 415

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGIGAYGARG CWTGIGGITG YCAYTAYGG

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

AAGCCTGGSA YICKYTTIGC CCAIYTIAT

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

TGTTACGAAG TGGGAATGGT GA

22

10

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: cDNA

20

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

TTGACACCAG ACCAACTGGT AATG

24

25

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

30

(ii) MOLECULE TYPE: cDNA

35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GGTGGCGACG ACTCCTGGAG CCCG

24

(2) INFORMATION FOR SEQ ID NO: 12:

5

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 22 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTACACTGAT TTGTAGCTGG AC

22

(2) INFORMATION FOR SEQ ID NO: 13:

20

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CCATGATGAT GTCCCTGACC

20

35

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

10 TCGCATGCCT GACGTGGGAC 20

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

15

(A) LENGTH: 24 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

25

GGCSTCCAGC ATCTCCAGSA RCAG 24

(2) INFORMATION FOR SEQ ID NO: 16:

30

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: cDNA



(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

GGAAGCTGGC TCACTTGCTG

20

5 (2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

10 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

TCTTGTTCTG GACAGGGATG

20

20

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

25 (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

35 GCATGGAACA TCTGCTCAAC

20

(2) INFORMATION FOR SEQ ID NO: 19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: cDNA

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

AGCAAGTTCA GCCTGTTAAG T

21

15

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1257 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

20

(ii) MOLECULE TYPE: cDNA

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:

ATGAATTACA GCATTCCCAG CAATGTCACT AACTTGGAAG GTGGGCCTGG TCGGCAGACC 60

30

ACAAGCCCAA ATGTGTTGTG GCCAACACCT GGGCACCTTT CTCCTTTAGT GGTCCATCGC 120

CAGTTATCAC ATCTGTATGC GGAACCTCAA AAGAGTCCCT GGTGTGAAGC AAGATCGCTA 180

GAACACACCT TACCTGTAAA CAGAGAGACA CTGAAAAGGA AGGTTAGTGG GAACCGTTGC 240

35

GCCAGCCCTG TTA CTGGTCC AGGTTCAAAG AGGGATGCTC ACTTCTGCGC TGTCTGCAGC 300

GATTACGCAT CGGGATATCA CTATGGAGTC TGGTCGTGTG AAGGATGTAA GGCCTTTTTT 360

AAAAGAAGCA TTCAAGGACA TAATGATTAT ATTTGTCCAG CTACAAATCA GTGTACAATC 420

GATAAAAACC GGC GCAAGAG CTGCCAGGCC TGCCGACTTC GGAAGTGTTA CGAAGTGGGA 480

5 ATGGTGAAGT GTGGCTCCCG GAGAGAGAGA TGTGGGTACC GCCTTGTGCG GAGACAGAGA 540

AGTGCCGACG AGCAGCTGCA CTGTGCCGGC AAGGCCAAGA GAAGTGGCGG CCACGCGCCC 600

CGAGTGCGGG AGCTGCTGCT GGACGCCCTG AGCCCCGAGC AGCTAGTGCT CACCCTCCTG 660

10 GAGGCTGAGC CGCCCCATGT GCTGATCAGC CGCCCCAGTG CGCCCTTCAC CGAGGCCTCC 720

ATGATGATGT CCCTGACCAA GTTGGCCGAC AAGGAGTTGG TACACATGAT CAGCTGGGCC 780

15 AAGAAGATTC CCGGCTTTGT GGAGCTCAGC CTGTTTCGACC AAGTGCGGCT CTTGGAGAGC 840

TGTTGGATGG AGGTGTTAAT GATGGGGCTG ATGTGGCGCT CAATTGACCA CCCC GGCAAG 900

CTCATCTTTG CTCCAGATCT TGTTCCTGGAC AGGGATGAGG GGAAATGCGT AGAAGGAATT 960

20 CTGGAAATCT TTGACATGCT CCTGGCAACT ACTTCAAGGT TTCGAGAGTT AAACTCCAA 1020

CACAAAGAAT ATCTCTGTGT CAAGGCCATG ATCCTGCTCA ATTCCAGTAT GTACCCCTCTG 1080

25 GTCACAGCGA CCCAGGATGC TGACAGCAGC CGGAAGCTGG CTCACTTGCT GAACGCCGTG 1140

ACCGATGCTT TGGTTTGGGT GATTGCCAAG AGCGGCATCT CCTCCCAGCA GCAATCCATG 1200

CGCCTGGCTA ACCTCCTGAT GCTCCTGTCC CACGTCAGGC ATGCGAGGTC TGCCTGA 1257

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 418 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

5

Met Asn Tyr Ser Ile Pro Ser Asn Val Thr Asn Leu Glu Gly Gly Pro  
1 5 10 15

10

Gly Arg Gln Thr Thr Ser Pro Asn Val Leu Trp Pro Thr Pro Gly His  
20 25 30

Leu Ser Pro Leu Val Val His Arg Gln Leu Ser His Leu Tyr Ala Glu  
35 40 45

15

Pro Gln Lys Ser Pro Trp Cys Glu Ala Arg Ser Leu Glu His Thr Leu  
50 55 60

Pro Val Asn Arg Glu Thr Leu Lys Arg Lys Val Ser Gly Asn Arg Cys  
65 70 75 80

20

Ala Ser Pro Val Thr Gly Pro Gly Ser Lys Arg Asp Ala His Phe Cys  
85 90 95

25

Ala Val Cys Ser Asp Tyr Ala Ser Gly Tyr His Tyr Gly Val Trp Ser  
100 105 110

Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser Ile Gln Gly His Asn  
115 120 125

30

Asp Tyr Ile Cys Pro Ala Thr Asn Gln Cys Thr Ile Asp Lys Asn Arg  
130 135 140

Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys Cys Tyr Glu Val Gly  
145 150 155 160

35

Met Val Lys Cys Gly Ser Arg Arg Glu Arg Cys Gly Tyr Arg Leu Val  
165 170 175

Arg Arg Gln Arg Ser Ala Asp Glu Gln Leu His Cys Ala Gly Lys Ala

180

185

190

Lys Arg Ser Gly Gly His Ala Pro Arg Val Arg Glu Leu Leu Leu Asp  
195 200 205

5

Ala Leu Ser Pro Glu Gln Leu Val Leu Thr Leu Leu Glu Ala Glu Pro  
210 215 220

10

Pro His Val Leu Ile Ser Arg Pro Ser Ala Pro Phe Thr Glu Ala Ser  
225 230 235 240

Met Met Met Ser Leu Thr Lys Leu Ala Asp Lys Glu Leu Val His Met  
245 250 255

15

Ile Ser Trp Ala Lys Lys Ile Pro Gly Phe Val Glu Leu Ser Leu Phe  
260 265 270

Asp Gln Val Arg Leu Leu Glu Ser Cys Trp Met Glu Val Leu Met Met  
275 280 285

20

Gly Leu Met Trp Arg Ser Ile Asp His Pro Gly Lys Leu Ile Phe Ala  
290 295 300

25

Pro Asp Leu Val Leu Asp Arg Asp Glu Gly Lys Cys Val Glu Gly Ile  
305 310 315 320

Leu Glu Ile Phe Asp Met Leu Leu Ala Thr Thr Ser Arg Phe Arg Glu  
325 330 335

30

Leu Lys Leu Gln His Lys Glu Tyr Leu Cys Val Lys Ala Met Ile Leu  
340 345 350

Leu Asn Ser Ser Met Tyr Pro Leu Val Thr Ala Thr Gln Asp Ala Asp  
355 360 365

35

Ser Ser Arg Lys Leu Ala His Leu Leu Asn Ala Val Thr Asp Ala Leu  
370 375 380

Val Trp Val Ile Ala Lys Ser Gly Ile Ser Ser Gln Gln Gln Ser Met

385                      390                      395                      400

Arg Leu Ala Asn Leu Leu Met Leu Leu Ser His Val Arg His Ala Arg

                         405                      410                      415

5

Ser Ala

(2) INFORMATION FOR SEQ ID NO: 22:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 34 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

CTTGGATCCA TAGCCCTGCT GTGATGAATT ACAG 34

(2) INFORMATION FOR SEQ ID NO: 23:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 33 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

GATGGATCCT CACCTCAGGG CCAGGCGTCA CTG 33

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1898 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

20 CACGAATCTT TGAGAACATT ATAATGACCT TTGTGCCTCT TCTTGCAAGG TGTTTTCTCA 60  
GCTGTTATCT CAAGACATGG ATATAAAAAA CTCACCATCT AGCCTTAATT CTCCTTCCTC 120  
25 CTACAACTGC AGTCAATCCA TCTTACCCCT GGAGCACGGC TCCATATACA TACCTTCCTC 180  
CTATGTAGAC AGCCACCATG AATATCCAGC CATGACATTC TATAGCCCTG CTGTGATGAA 240  
TTACAGCATT CCCAGCAATG TCACTAACTT GGAAGGTGGG CCTGGTCGGC AGACCACAAG 300  
CCCAAATGTG TTGTGGCCAA CACCTGGGCA CCTTTCTCCT TTAGTGGTCC ATCGCCAGTT 360  
ATCACATCTG TATGCGGAAC CTCAAAGAG TCCCTGGTGT GAAGCAAGAT CGCTAGAACA 420  
30 CACCTTACCT GTAAACAGAG AGACACTGAA AAGGAAGGTT AGTGGGAACC GTTGCGCCAG 480  
CCCTGTTACT GGTCCAGGTT CAAAGAGGGA TGCTCACTTC TCGCTGTCT GCAGCGATTA 540  
CGCATCGGGA TATCACTATG GAGTCTGGTC GTGTGAAGGA TGTAAGGCCT TTTTAAAAAG 600  
AAGCATTCAA GGACATAATG ATTATATTTG TCCAGCTACA AATCAGTGTA CAATCGATAA 660  
AAACCGGCGC AAGAGCTGCC AGGCCTGCCG ACTTCGGAAG TGTTACGAAG TGGGAATGGT 720

GAAGTGTGGC TCCCGGAGAG AGAGATGTGG GTACCGCCTT GTGCGGAGAC AGAGAAGTGC 780

CGACGAGCAG CTGCACTGTG CCGGCAAGGC CAAGAGAAGT GGC GGCCACG CGCCCCGAGT 840

5 GCGGGAGCTG CTGCTGGACG CCCTGAGCCC CGAGCAGCTA GTGCTCACCC TCCTGGAGGC 900

TGAGCCGCCC CATGTGCTGA TCAGCCGCCC CAGTGCGCCC TTCACCGAGG CCTCCATGAT 960

10 GATGTCCCTG ACCAAGTTGG CCGACAAGGA GTTGGTACAC ATGATCAGCT GGGCCAAGAA 1020

GATTCCCGGC TTTGTGGAGC TCAGCCTGTT CGACCAAGTG CGGCTCTTGG AGAGCTGTTG 1080

GATGGAGGTG TTAATGATGG GGCTGATGTG GCGCTCAATT GACCACCCCG GCAAGCTCAT 1140

15 CTTTGCTCCA GATCTTGTTT TGGACAGGGA TGAGGGGAAA TGCGTAGAAG GAATTCTGGA 1200

AATCTTTGAC ATGCTCCTGG CAACTACTTC AAGGTTTCGA GAGTTAAAC TCCAACACAA 1260

20 AGAATATCTC TGTGTCAAGG CCATGATCCT GCTCAATTCC AGTATGTACC CTCTGGTCAC 1320

AGCGACCCAG GATGCTGACA GCAGCCGGAA GCTGGCTCAC TTGCTGAACG CCGTGACCGA 1380

TGCTTTGGTT TGGGTGATTG CCAAGAGCGG CATCTCCTCC CAGCAGCAAT CCATGCGCCT 1440

25 GGCTAACCTC CTGATGCTCC TGTCCACGT CAGGCATGCG AGTAACAAGG GCATGGAACA 1500

TCTGCTCAAC ATGAAGTGCA AAAATGTGGT CCCAGTGTAT GACCTGCTGC TGGAGATGCT 1560

30 GAATGCCCAC GTGCTTCGCG GGTGCAAGTC CTCCATCACG GGGTCCGAGT GCAGCCCGGC 1620

AGAGGACAGT AAAAGCAAAG AGGGCTCCCA GAACCCACAG TCTCAGTGAC GCCTGGCCCT 1680

GAGGTGAACT GGCCACAGA GGTCACAAGC TGAAGCGTGA ACTCCAGTGT GTCAGGAGCC 1740

35 TGGGCTTCAT CTTTCTGCTG TGTGGTCCCT CATTTGGTGA TGGCAGGCTT GGTCATGTAC 1800

CATCCTTCCC TCCACCTTCC CAACTCTCAG GAGTCGGTGT GAGGAAGCCA TAGTTTCCCT 1860



TGTTAGCAGA GGGACATTTG AATCGAGCGT TTCCACAC

1898

(2) INFORMATION FOR SEQ ID NO: 25:

5

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 530 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: peptide

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

Met Asp Ile Lys Asn Ser Pro Ser Ser Leu Asn Ser Pro Ser Ser Tyr  
1 5 10 15

20

Asn Cys Ser Gln Ser Ile Leu Pro Leu Glu His Gly Ser Ile Tyr Ile  
20 25 30

25

Pro Ser Ser Tyr Val Asp Ser His His Glu Tyr Pro Ala Met Thr Phe  
35 40 45

Tyr Ser Pro Ala Val Met Asn Tyr Ser Ile Pro Ser Asn Val Thr Asn  
50 55 60

30

Leu Glu Gly Gly Pro Gly Arg Gln Thr Thr Ser Pro Asn Val Leu Trp  
65 70 75 80

Pro Thr Pro Gly His Leu Ser Pro Leu Val Val His Arg Gln Leu Ser  
85 90 95

35

His Leu Tyr Ala Glu Pro Gln Lys Ser Pro Trp Cys Glu Ala Arg Ser  
100 105 110

Leu Glu His Thr Leu Pro Val Asn Arg Glu Thr Leu Lys Arg Lys Val

115 120 125

Ser Gly Asn Arg Cys Ala Ser Pro Val Thr Gly Pro Gly Ser Lys Arg  
130 135 140

5 Asp Ala His Phe Cys Ala Val Cys Ser Asp Tyr Ala Ser Gly Tyr His  
145 150 155 160

10 Tyr Gly Val Trp Ser Cys Glu Gly Cys Lys Ala Phe Phe Lys Arg Ser  
165 170 175

Ile Gln Gly His Asn Asp Tyr Ile Cys Pro Ala Thr Asn Gln Cys Thr  
180 185 190

15 Ile Asp Lys Asn Arg Arg Lys Ser Cys Gln Ala Cys Arg Leu Arg Lys  
195 200 205

Cys Tyr Glu Val Gly Met Val Lys Cys Gly Ser Arg Arg Glu Arg Cys  
210 215 220

20 Gly Tyr Arg Leu Val Arg Arg Gln Arg Ser Ala Asp Glu Gln Leu His  
225 230 235 240

Cys Ala Gly Lys Ala Lys Arg Ser Gly Gly His Ala Pro Arg Val Arg  
245 250 255

25 Glu Leu Leu Leu Asp Ala Leu Ser Pro Glu Gln Leu Val Leu Thr Leu  
260 265 270

30 Leu Glu Ala Glu Pro Pro His Val Leu Ile Ser Arg Pro Ser Ala Pro  
275 280 285

Phe Thr Glu Ala Ser Met Met Met Ser Leu Thr Lys Leu Ala Asp Lys  
290 295 300

35 Glu Leu Val His Met Ile Ser Trp Ala Lys Lys Ile Pro Gly Phe Val  
305 310 315 320

Glu Leu Ser Leu Phe Asp Gln Val Arg Leu Leu Glu Ser Cys Trp Met

325

330

335

Glu Val Leu Met Met Gly Leu Met Trp Arg Ser Ile Asp His Pro Gly  
340 345 350

5

Lys Leu Ile Phe Ala Pro Asp Leu Val Leu Asp Arg Asp Glu Gly Lys  
355 360 365

10

Cys Val Glu Gly Ile Leu Glu Ile Phe Asp Met Leu Leu Ala Thr Thr  
370 375 380

Ser Arg Phe Arg Glu Leu Lys Leu Gln His Lys Glu Tyr Leu Cys Val  
385 390 395 400

15

Lys Ala Met Ile Leu Leu Asn Ser Ser Met Tyr Pro Leu Val Thr Ala  
405 410 415

Thr Gln Asp Ala Asp Ser Ser Arg Lys Leu Ala His Leu Leu Asn Ala  
420 425 430

20

Val Thr Asp Ala Leu Val Trp Val Ile Ala Lys Ser Gly Ile Ser Ser  
435 440 445

25

Gln Gln Gln Ser Met Arg Leu Ala Asn Leu Leu Met Leu Leu Ser His  
450 455 460

Val Arg His Ala Ser Asn Lys Gly Met Glu His Leu Leu Asn Met Lys  
465 470 475 480

30

Cys Lys Asn Val Val Pro Val Tyr Asp Leu Leu Leu Glu Met Leu Asn  
485 490 495

Ala His Val Leu Arg Gly Cys Lys Ser Ser Ile Thr Gly Ser Glu Cys  
500 505 510

35

Ser Pro Ala Glu Asp Ser Lys Ser Lys Glu Gly Ser Gln Asn Pro Gln  
515 520 525

Ser Gln

530

(2) INFORMATION FOR SEQ ID NO: 26:

5 (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

10

(ii) MOLECULE TYPE: other nucleic acid

15

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GTGCGGATCC TCTCAAGACA TGGATATAAA

30

20

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: other nucleic acid

30

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

35

AGTAACAGGG CTGGCGCAAC GGTTT

25

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

5

(ii) MOLECULE TYPE: other nucleic acid

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

ACTGGCGATG GACCACTAAA GG

22

15

ACTGGCGATG GACCACTAAA GG

**Claims:**

1. Isolated DNA encoding a protein having an N-terminal domain, a DNA-binding domain and a ligand-binding domain, wherein the amino acid sequence of said DNA-binding domain of said protein exhibits at least 80% homology with the amino acid sequence shown in SEQ ID NO:3, and the amino acid sequence of said ligand-binding domain of said protein exhibits at least 70% homology with the amino acid sequence shown in SEQ ID NO:4.
2. Isolated DNA according to claims 1, characterized in that the amino acid sequence of said DNA-binding domain of said protein exhibits at least 90%, preferably 95%, more preferably 98%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:3.
3. Isolated DNA according to claims 1 or 2, characterized in that the amino acid sequence of said ligand-binding domain of said protein exhibits at least 75%, preferably 80%, more preferably 90%, most preferably 100% homology with the amino acid sequence shown in SEQ ID NO:4.
4. Isolated DNA according to claims 1 to 3, said DNA encoding a protein comprising the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.
5. Isolated DNA according to claims 1 to 4, characterized in that said DNA comprises the nucleic acid sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:20 or SEQ ID NO:24.
6. A recombinant expression vector comprising the DNA according to any of the claims 1 to 5.

7. A cell transfected with DNA according to claims 1 to 5 or an expression vector according to claim 6.
8. A cell according to claim 7 which is a stable transfected cell line which expresses the steroid receptor protein according to any of the claims 9 to 11.
9. Protein encoded by DNA according to claims 1 to 5 or an expression vector according to claim 6.
10. Protein according to claim 9, said protein comprising the amino acid sequence of SEQ ID NO:5, SEQ ID NO:6, SEQ ID NO:21 or SEQ ID NO:25.
11. Chimeric protein having an N-terminal domain, a DNA-binding domain, and a ligand-binding domain, characterized in that at least one of said domains of said chimeric protein originates from a protein according to claims 9 or 10, and at least one of the other domains of said chimeric protein originates from another receptor protein from the nuclear receptor superfamily, provided that the DNA-binding domain and the ligand-binding domain of said chimeric protein originates from different proteins.
12. DNA encoding a protein according to claim 11.
13. Use of a DNA according to claims 1 to 5 or 12, an expression vector according to claim 6, a cell according to claim 7 or 8 or a protein according to claim 9 to 11 in a screening assay for identification of new drugs.

14. A method for identifying functional ligands for the protein according to claims 9 to 11, said method comprising the steps of

- a) introducing into a suitable host cell 1) DNA according to claims 1 to 5 or 12, and 2) a suitable reporter gene functionally linked to an operative hormone response element, said HRE being able to be activated by the DNA-binding domain of the protein encoded by said DNA;
- b) bringing the host cell from step a) into contact with potential ligands which will possibly bind to the ligand-binding domain of the protein encoded by said DNA from step a);
- c) monitoring the expression of the protein encoded by said reporter gene of step a).



## ABSTRACT

The present invention relates to isolated DNA encoding novel estrogen receptors, the proteins encoded by said DNA, chimeric receptors comprising parts of said novel receptors and uses thereof.

5

169250 "T" 92 92880

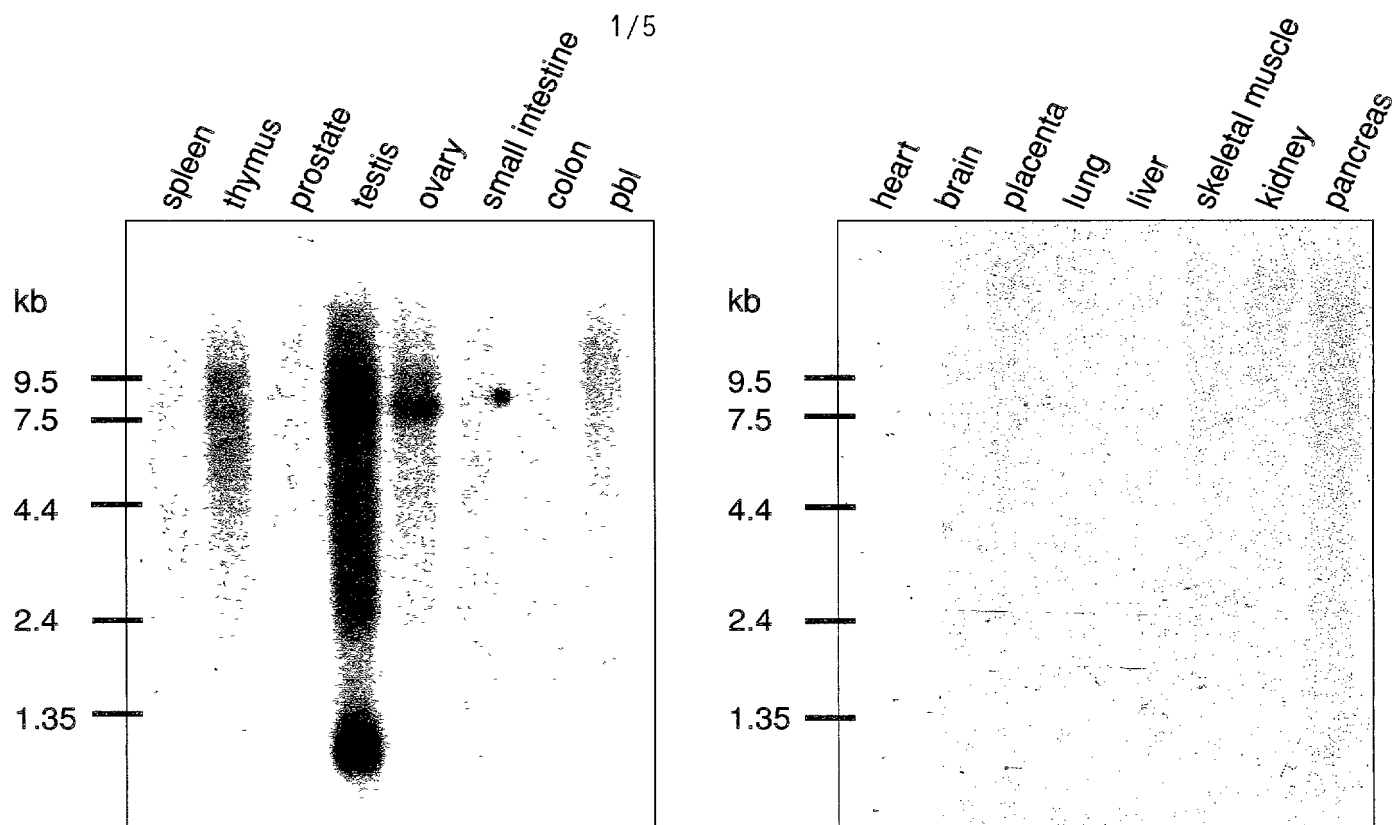


Figure 1

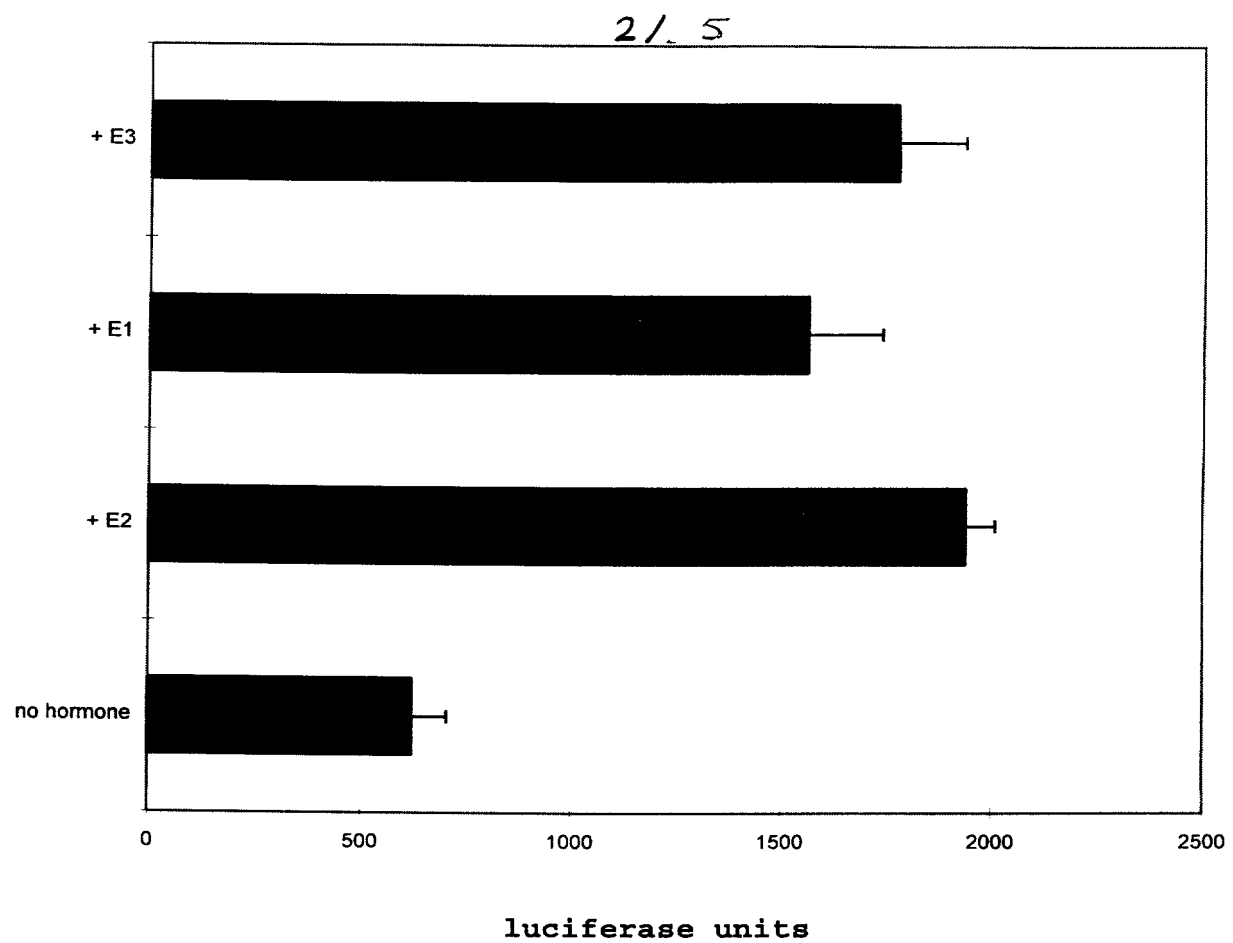


Fig. 2

Transient transfection of CHO cells with Estrogen Receptors Alpha and Beta  
Incubation with Estradiol and ICI

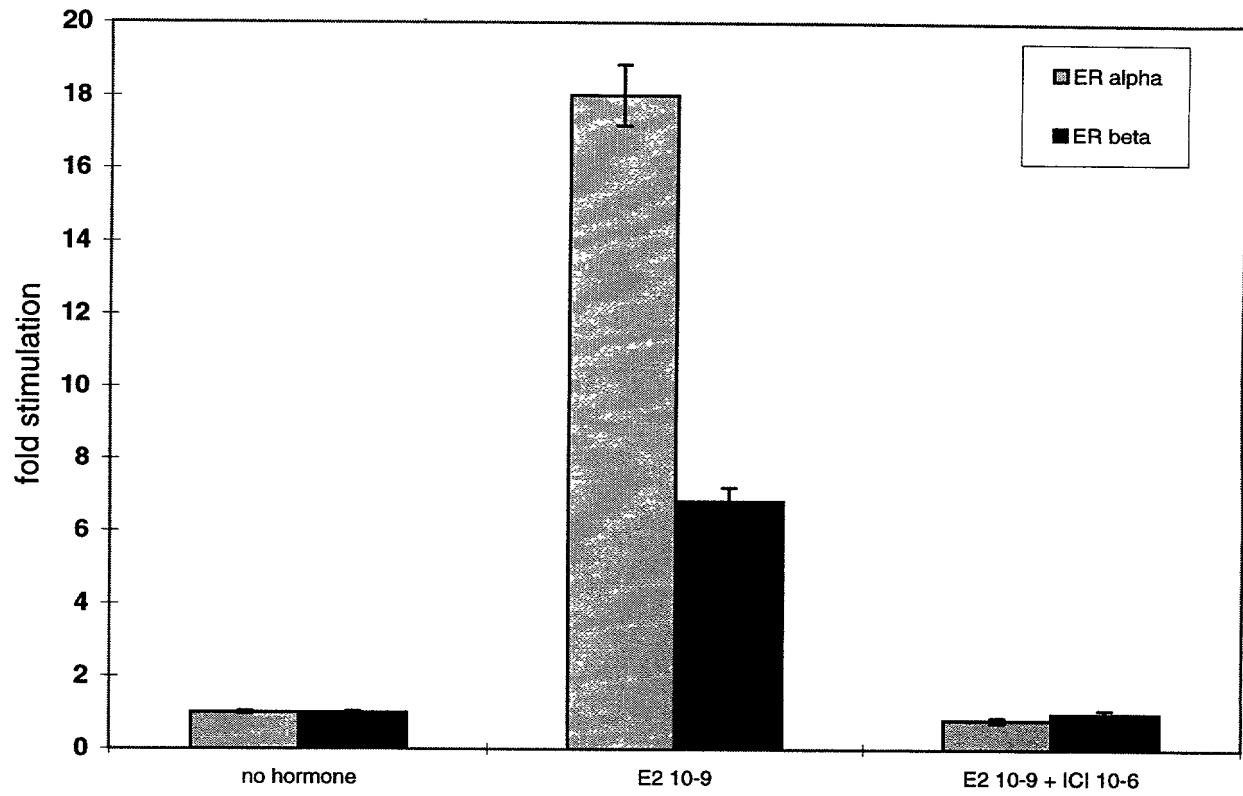
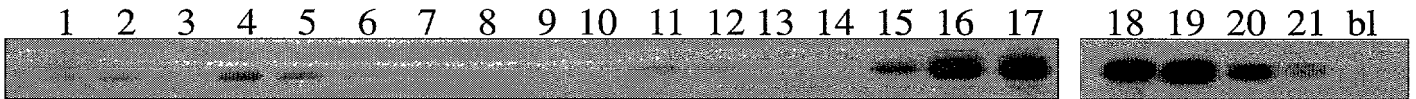


Figure 3

ER $\alpha$  and ER $\beta$  RT PCR on tissue-representative cell linesER $\alpha$ ER $\beta$ 

- |             |           |             |              |           |
|-------------|-----------|-------------|--------------|-----------|
| 1. Ishikawa | 6. HOS    | 11. Hs-760T | 16. HUV-EC-C | 21. RASMC |
| 2. HEC-1A   | 7. U2-OS  | 12. SW-954  | 17. BAEC-1   | bl. blank |
| 3. RL95-2   | 8. MG-63  | 13. Hep- G2 | 18. A10      |           |
| 4. ECC-1    | 9. MCF-7  | 14. CaCo    | 19. A7R5     |           |
| 5. SaOS-2   | 10. T47-D | 15. HISM    | 20. CavaSMC  |           |

Figure 4

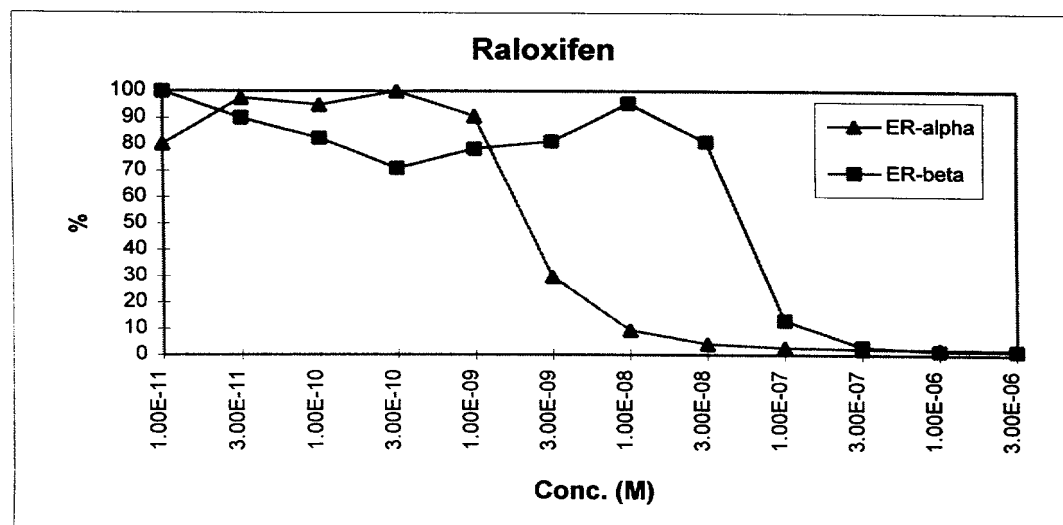
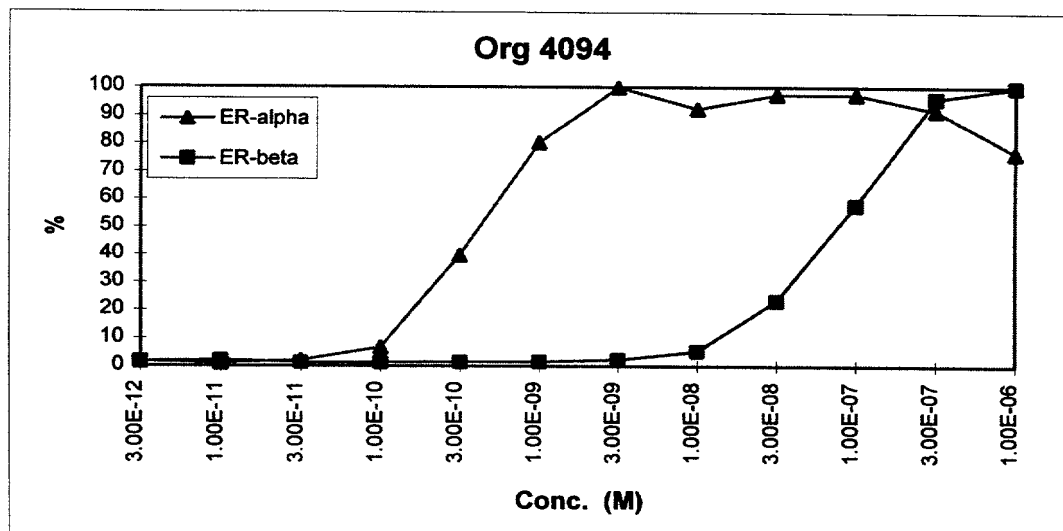
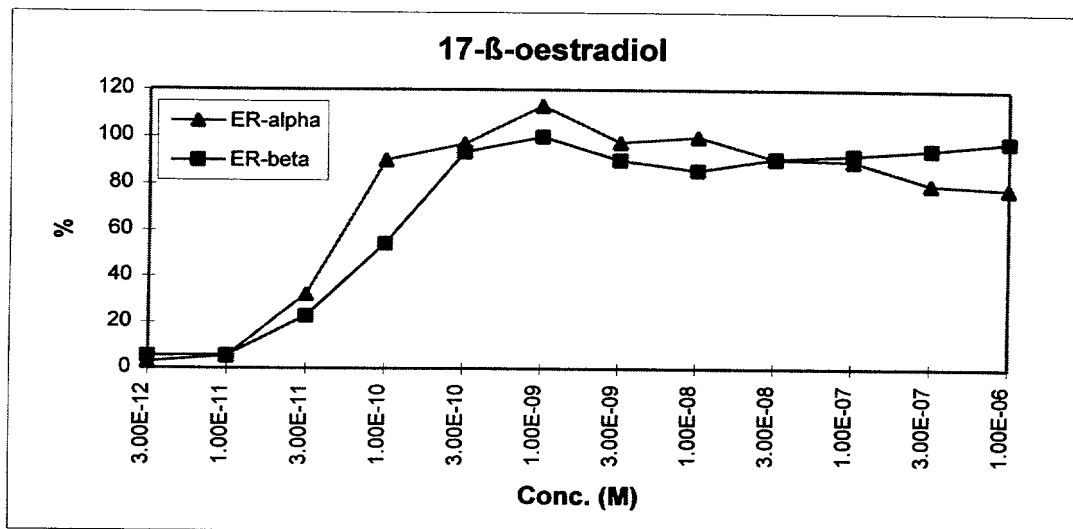


Figure 5